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The Hydrophobic Cysteine-rich Domain of SNAP25 Couples with Downstream Residues to Mediate Membrane Interactions and Recognition by DHHC Palmitoyl Transferases

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SNAP25 is synthesized as a soluble protein but must associate with the plasma membrane to function in exocytosis; however, this membrane-targeting pathway is poorly defined. SNAP25 contains a palmitoylated cysteine-rich domain with four cysteines, and we show that coexpression of specific DHHC palmitoyl transferases is sufficient to promote SNAP25 membrane association in HEK293 cells. siRNA-mediated knockdown of its SNARE partner, syntaxin 1A, does not affect membrane interaction of SNAP25 in PC12 cells, whereas specific cysteine-to-alanine mutations perturb membrane binding, which is restored by leucine substitutions. These results suggest a role for cysteine hydrophobicity in initial membrane interactions of SNAP25, and indeed other hydrophobic residues in the cysteine-rich domain are also important for membrane binding. In addition to the cysteine-rich domain, proline-117 is also essential for SNAP25 membrane binding, and experiments in HEK293 cells revealed that mutation of this residue inhibits membrane binding induced by coexpression with DHHC17, but not DHHC3 or DHHC7. These results suggest a model whereby SNAP25 interacts autonomously with membranes via its hydrophobic cysteine-rich domain, requiring only sufficient expression of partner DHHC proteins for stable membrane binding. The role of proline-117 in SNAP25 palmitoylation is one of the first descriptions of elements within substrate proteins that modulate DHHC specificity.

INTRODUCTION

The posttranslational thioester linkage of palmitate groups onto cysteine residues (S-palmitoylation) plays an important role in regulating protein interactions with intracellular membranes. In addition, palmitoylation can regulate the intracellular trafficking of proteins, protein microlocalization within membranes, and protein stability (Resh, 2006; Greaves and Chamberlain, 2007; Linder and Deschenes, 2007; Nadolski and Linder, 2007). The study of protein palmitoylation has been severely hindered in the past due to a lack of knowledge about the palmitoylating enzymes. However, 23 mammalian and seven yeast palmitoyl transferases (PATs) containing a signature DHHC-CRD (cysteine-rich domain) were recently identified (Lobo *et al.*, 2002; Roth *et al.*, 2002; Fukata *et al.*, 2004; Huang *et al.*, 2004; Keller *et al.*, 2004; Mitchell *et al.*, 2006) and shown to be responsible for the large majority of cellular palmitoylation, in yeast at least (Roth *et al.*, 2006). Mutation of residues in the DHHC domain of these enzymes blocks activity, suggesting that this region of the proteins may form part of the catalytic site.

DHHC proteins have four or more predicted transmembrane domains (TMDs), with the DHHC-CRD putative catalytic domain predicted to be cytosolically exposed; this topology has been confirmed for the yeast enzyme Akr1p (Politis *et al.*, 2005). The large number of DHHC proteins and the association of several of these proteins with the same intracellular compartments (predominantly ER/Golgi) suggest likely differences in substrate specificity and regulation. Indeed, some yeast enzymes were shown to exhibit preferences for certain types of substrate, and although some proteins were modified by more than one enzyme, others required a specific enzyme for palmitoylation (Hou *et al.*, 2005; Smotrys *et al.*, 2005; Roth *et al.*, 2006). The identification of the DHHC family of PATs has reinvigorated the study of protein palmitoylation and provides essential information and tools to dissect the outcome(s) of protein palmitoylation in a cellular context.

Although no general consensus sequence specifying palmitoylation exists, a compelling factor that decides whether

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Abbreviations used: CSP, cysteine-string protein; DHHC, aspartic acid-histidine-histidine-cysteine motif; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; HEK, human embryonic kidney; PAT, palmitoyl transferase; PC12, pheochromocytoma-12; SNAP25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor.

a cysteine residue is palmitoylated is undoubtedly its proximity to the membrane. As previously discussed, the exclusive membrane localization of DHHC proteins implies that palmitoylated proteins require specific membrane-targeting signals to facilitate initial membrane interaction (Greaves and Chamberlain, 2006, 2007; Greaves *et al.*, 2008). A number of studies have highlighted the importance of palmitoylation for correct sorting of proteins containing TMDs, e.g., (Hayashi *et al.*, 2005; Lam *et al.*, 2006; Abrami *et al.*, 2008). In addition to TMD proteins, a number of signaling molecules utilize isoprenyl or myristoyl modifications (which are added in the cytosol) to mediate transient membrane interactions. For example, the farnesyl group of H- and N-Ras provides the proteins with a weak membrane affinity that allows the protein to "sample" a variety of intracellular membranes (Magee *et al.*, 1987; Choy *et al.*, 1999; Goodwin *et al.*, 2005; Rocks *et al.*, 2005). Palmitoylation only occurs when Ras associates with a membrane compartment (Golgi/ER) containing the Ras PAT (Swarthout *et al.*, 2005); palmitoylation "traps" Ras on that membrane, facilitating its forward transport (Rocks *et al.*, 2005; Roy *et al.*, 2005).

While primary membrane-targeting information in some proteins is obvious, such as TMDs or isoprenyl/myristoyl modifications, other palmitoylated proteins lack obvious membrane-targeting signals (Greaves and Chamberlain, 2006). This is the case with SNAP25, an essential component of the neuronal/neuroendocrine SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) complex that mediates exocytosis. In contrast to the large majority of SNAREs, SNAP25 lacks a TMD, and mutations within the palmitoylated cysteine-rich domain have been reported to block stable membrane association and targeting (Vogel *et al.*, 2000; Washbourne *et al.*, 2001). The mechanisms regulating SNAP25 membrane targeting before palmitoylation have been much debated. In particular, some studies have suggested a key role for syntaxin 1 (the SNARE partner of SNAP25) in driving initial membrane interaction of SNAP25 (Vogel *et al.*, 2000; Washbourne *et al.*, 2001); however, others (Gonzalo *et al.*, 1999; Loranger and Linder, 2002) have refuted this idea. In particular, a minimal fragment of SNAP25 containing amino acids 85-120 (which includes the palmitoylated cysteines) was shown to target enhanced green fluorescent protein (EGFP) to the plasma membrane (Gonzalo *et al.*, 1999). This region of SNAP25 lacks the SNARE motifs and hence syntaxin-binding sites, providing evidence that SNAP25 traffics independently of syntaxin (Loranger and Linder, 2002). In addition, a recent study reported that syntaxin 1A perturbed plasma membrane delivery of SNAP25 when expressed in the absence of the syntaxin 1A chaperone, munc18 (Medine *et al.*, 2007).

To probe the mechanisms involved in SNAP25 membrane interactions further, we have performed a detailed mutagenic study of the 85-120 minimal membrane-targeting domain present within *full-length* SNAP25 and examined how specific DHHC enzymes regulate SNAP25 membrane interaction. Cysteine residues and specific surrounding amino acids are implicated in initial membrane binding, most likely via hydrophobic interactions with membranes. Initial access to the membrane interface is important to allow interaction with specific DHHC proteins, which palmitoylate SNAP25 and promote stable membrane attachment. Interestingly, conserved amino acids (in particular proline-117) appear to be important in determining the specificity of DHHC interaction.

MATERIALS AND METHODS

Plasmid Constructs, Antibodies, and Chemicals

Plasmid containing rat SNAP25B fused to an N-terminal EGFP tag was as previously described (Greaves *et al.*, 2008). This construct (lacking the initiating ATG of SNAP25B) was used as a template to produce all the SNAP25B substitution and deletion mutants used in this study by site-directed mutagenesis. This plasmid was also used as a template to amplify the nucleotide sequence coding for amino acids 93-120, which was then cloned into pEGFPC2 using complimentary restriction sites that were present within the oligonucleotide primers used in the PCR reaction. SNAP25B(85-120) with a C-terminal EGFP tag was kindly provided by Dr. Maurine Linder (Washington University at St. Louis) (Gonzalo *et al.*, 1999). Hemagglutinin (HA)-tagged mouse DHHC3, DHHC7, and DHHC17 clones in pEFBOS-HA were as previously described (Fukata *et al.*, 2004). DHHC-to-DHHS mutations were introduced into the respective plasmids by site-directed mutagenesis (Greaves *et al.*, 2008). The fidelity of all mutant constructs was confirmed by DNA sequencing (University of Dundee DNA sequencing service, Dundee, Scotland).

Anti-GFP mAb (JL8) was purchased from Clontech (Palo Alto, CA). Anti-SNAP25 and anti-syntaxin 1A antibodies were supplied by Synaptic Systems (Göttingen, Germany). Anti-HA mAb and COMPLETE protease inhibitor cocktail were from Roche (Lewes, East Sussex, United Kingdom). Subcellular proteome extraction kit (SPEK) was purchased from Merck Biosciences (Nottingham, United Kingdom). All other reagents were of an analytical grade from Sigma (Poole, Dorset, United Kingdom).

Cell Culture and Cell Transfection

PC12 cells were grown in RPMI1640 media with 10% horse serum and 5% fetal calf serum containing penicillin/streptomycin. Human embryonic kidney (HEK293) cells were cultured in DMEM with 10% fetal calf serum with penicillin/streptomycin. All reagents used for maintenance of cells were purchased from Invitrogen (Paisley, United Kingdom). Cells were maintained in a humidified atmosphere containing 5% CO_2.

For all experiments, cells were plated onto 24-well plates or coverslips precoated with poly-D-lysine. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions; the ratio of lipofectamine to DNA used was 2:1. PC12 cells were analyzed ~40 h after transfection, and HEK293 cells were used ~20 h after transfection.

Subcellular Fractionation

Cells were fractionated into cytosol, and membrane fractions using selected buffers from an SPEK kit (Merck), which isolates defined cell fractions by differential detergent extraction (Ramsby *et al.*, 1994; Greaves and Chamberlain, 2006; Greaves *et al.*, 2008). Briefly, cells were washed 2× in PBS and then incubated on ice in 150 μl of buffer 1 containing protease inhibitors for 10 min. The buffer was then removed and centrifuged at 2000 × *g* to remove cell debris; this fraction contained cell cytosol. The remaining cell material was solubilized in SDS-dissociation buffer. As an alternative fractionation procedure, cells were homogenized in HES buffer (20 mM HEPES, 1 mM EDTA, and 250 mM sucrose, pH 7.4) using a Dounce homogenizer. The homogenate was centrifuged at 190,000 × *g* for 30 min to separate cytosol (supernatant) and membrane (pellet) fractions. Comparison of this method of cell fractionation to the SPEK procedure confirmed the validity of the SPEK approach for cell fractionation (Supplementary Figure S1).

Small Interfering RNA Transfection

PC12 cells growing on poly-D-lysine coated 24-well plates were transfected with either 100 nM random small interfering RNA (siRNA) or a mixture of two siRNAs against syntaxin 1A (50 nM of each) using Dharmafect reagent. siRNAs and transfection reagent were supplied by Dharmacon Research (Boulder, CO). Approximately 70 h after transfection, the cells were lysed in SDS-dissociation buffer or fractionated using SPEK.

Palmitate Labeling

HEK293 cells plated on six-well plates were transfected with EGFP-SNAP25B or the SNAP25B(117-120A) mutant together with HA-DHHC3 or HA-DHHC17. Approximately 20 h after transfection, the cells were incubated with 0.5 mCi/ml [^{3}H]palmitic acid (Perkin Elmer-Cetus, Beaconsfield, United Kingdom) for 4 h, and EGFP-SNAP25B or the 117-120A mutant were subsequently immunoprecipitated using anti-GFP antibody coupled to magnetic microbeads (Miltenyi Biotech, Bisley, United Kingdom). The precipitated samples were resolved on duplicate gels and transferred to nitrocellulose membranes that were then either subjected to immunoblotting using anti-GFP or were exposed to film with the aid of a Kodak Biomax Transcreen LE intensifier screen (Eastman Kodak, Rochester, NY) for detection of [^{3}H]palmitate incorporation.

Immunofluorescence

PC12 cells growing on poly-D-lysine–coated coverslips were fixed in 4% formaldehyde (in PBS) for 30 min at room temperature, washed in PBS, and

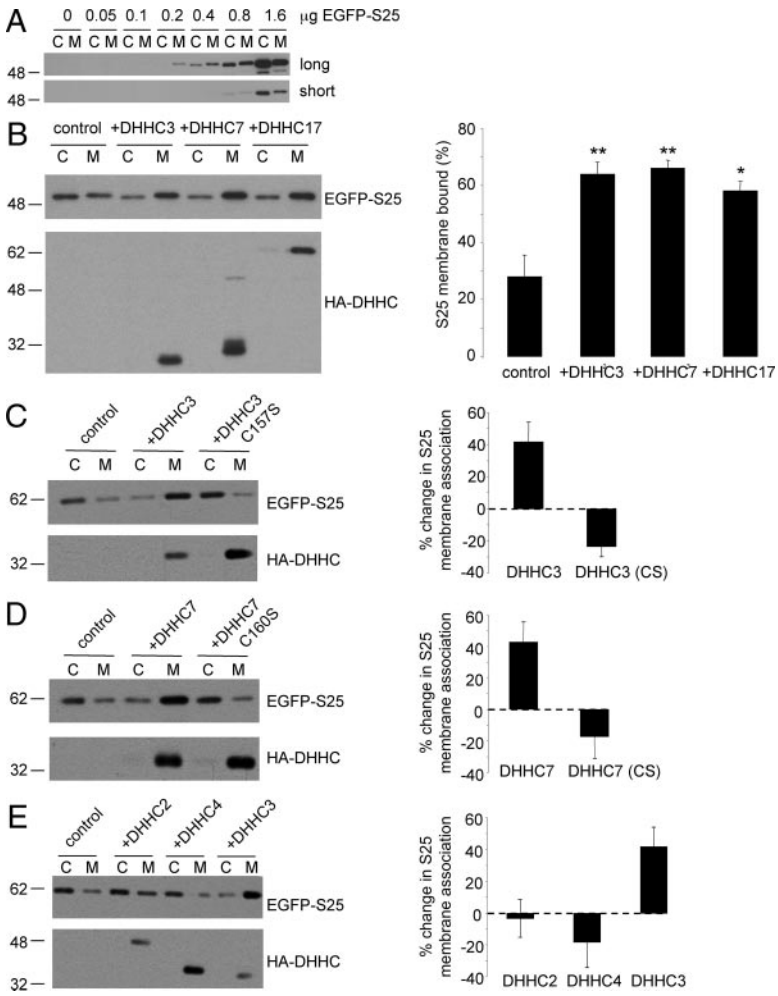

Figure 1. The extent of membrane binding of SNAP25B in HEK293 cells is dependent on expression levels and is enhanced by coexpression of specific DHHC palmitoyl transferases. (A) HEK293 cells were transfected with a range of amounts of EGFP-SNAP25B plasmid as indicated. The total amount of transfected DNA in each condition was kept constant by including empty pEGFPC2 vector in appropriate amounts. After ~20 h of transfection, the cells were fractionated into cytosol (C) and membrane (M) fractions. Distribution of SNAP25B in the recovered fractions was determined by immunoblotting with anti-GFP. Short and long denote short and long exposure times of the same immunoblot. (B) HEK293 cells were transfected with 0.8 μg EGFP-SNAP25B plasmid with or without 1.6 μg of HA-DHHC3, HA-DHHC7, or HA-DHHC17. Recovered cytosol (C) and membrane (M) fractions were analyzed by immunoblotting with anti-GFP and anti-HA. Left panel, representative immunoblots; right panel, average percentage membrane association of SNAP25B after cotransfection with the DHHCs compared with transfection in the absence of DHHC (n = 5). ** $p < 0.005$ and * $p < 0.02$, compared with the % membrane binding of EGFP-SNAP25B in the absence of DHHC expression. (C) EGFP-SNAP25B was transfected into HEK293 cells with or without HA-DHHC3 or HA-DHHC3(C157S) plasmids, and recovered cytosol (C) and membrane (M) fractions were analyzed by immunoblotting using anti-GFP and anti-HA antibodies. Left panel, representative immunoblots; right panel, average percentage change in membrane association of SNAP25B after cotransfection with the DHHCs compared with transfection in the absence of DHHC (n = 4). The effects of DHHC3 and DHHC3(C157S) cotransfection on SNAP25B membrane binding were significantly different ($p < 0.004$ using a Student's *t* test). (D) HEK293 cells transfected with EGFP-SNAP25B with or without DHHC7 or DHHC7(C160S) cotransfection were analyzed as described for panel C. The effects of DHHC7 and DHHC7(C160S) cotransfection on SNAP25B membrane binding were significantly different ($p < 0.01$ using a Student's *t* test). (E) HEK293 cells transfected with EGFP-SNAP25B with or without DHHC2, DHHC4, or DHHC3 were analyzed as described for panel C. The effects of DHHC2 and DHHC4 cotransfection on SNAP25B membrane binding were significantly different from DHHC3 cotransfection ($p < 0.03$ and $p < 0.02$, respectively, using a Student's *t* test). Position of molecular-weight standards is shown on the left side of all panels. Note that polyacrylamide gels used in A and B were 10% gels that are used to highlight the different migration of cytosolic and membrane-associated SNAP25B. In contrast, 4–12% precast gels were used in C–E; these gels do not resolve the different migrations of cytosolic and membrane-bound pools of SNAP25B. SNAP25B migrates at a slightly higher position on 4–12% gels than on 10% gels relative to the molecular-weight standards.

mounted onto slides using Mowiol 4-88 reagent. Imaging was performed using a Zeiss LSM 5 Pascal laser scanning microscope (Zeiss, Oberkochen, Germany).

Quantification and Statistical Analysis

Quantification of band density on immunoblots was determined using ImageJ software (http://rsb.info.nih.gov/ij/). Data are expressed as average % membrane association ± SEs. Statistical analysis was performed using unpaired Student's *t* test.

RESULTS

Membrane Binding of SNAP25B Is Inefficient in HEK293 Cells and Is Enhanced by Coexpression of Specific DHHC Palmitoyl Transferases

To probe the mechanisms involved in SNAP25 membrane interactions, we initially examined membrane binding of the protein in HEK293 cells, which lack neuronal-specific partner proteins of SNAP25. As a first step, we examined the distribution of EGFP-SNAP25B within cytosol and membrane fractions purified from transfected cells. This analysis demonstrated that SNAP25B was largely membrane associated at low expression levels (Figure 1A), in agreement with previous work showing efficient plasma membrane delivery of SNAP25 in HEK293 cells (Medine *et al.*, 2007). However, as expression levels of EGFP-SNAP25B were increased, there was a gradual loss of relative membrane association (Figure 1A). This suggests that a factor(s) required for membrane targeting of SNAP25B is present in only limiting concentrations in HEK293 cells, and this cell type thus provides a useful model to examine proteins that regulate SNAP25B membrane interaction.

We recently showed that membrane association of cysteine-string protein (CSP) in HEK293 cells is enhanced after coexpression of its partner DHHC proteins (DHHC3, DHHC7, DHHC15, or DHHC17; Greaves *et al.*, 2008). As SNAP25B is palmitoylated by the same enzymes as CSP (Fukata *et al.*, 2006), we reasoned that inefficient membrane association of SNAP25B in HEK293 cells may also reflect limiting expression of these DHHC proteins. To test this idea, we examined the effects on SNAP25B membrane inter-

action of coexpressing DHHC3, DHHC7, or DHHC17. All three DHHC proteins promoted a significant increase in the level of SNAP25B membrane binding (Figure 1B). This effect was specific, as mutations within the putative catalytic domains (DHHC to DHHS mutations) of DHHC3 and DHHC7 completely abolished the ability of the enzymes to enhance membrane binding of SNAP25B, and indeed both mutants decreased SNAP25B membrane association (Figure 1, C and D).

As a further test of DHHC specificity, we examined the effects on SNAP25B membrane binding of coexpressing DHHC2 or DHHC4, enzymes that are not known to palmitoylate SNAP25B (Fukata *et al.,* 2006). These enzymes had no stimulatory effect on SNAP25B membrane binding (Figure 1E), despite being expressed at similar levels to DHHC3. These results clearly demonstrate that specific palmitoyl transferases are sufficient to drive stable membrane interaction of SNAP25B in HEK293 cells.

Note that palmitoylation of SNAP25 leads to a decreased rate of migration by SDS-PAGE that is visible under specific gel conditions (Gonzalo and Linder, 1998). This band-shift was visible for EGFP-SNAP25B when samples were resolved on 10% SDS-PAGE gels (as shown in Figure 1, A and B). This observation shows that the increase in membrane binding of SNAP25B promoted by DHHC3/DHHC7/DHHC17 in HEK293 cells correlates with increased palmitoylation. The visualization of a palmitoylation-dependent band-shift in SNAP25 gives a more meaningful readout of palmitoylation status than [^{3}H]palmitate labeling. For example, it is not easy to determine whether a change in [^{3}H]palmitate incorporation into a multiply palmitoylated protein such as SNAP25 reflects: 1) an increase in the number of protein molecules that are palmitoylated, 2) an increased extent of palmitoylation of a fixed pool of protein, or 3) a combination of these factors. By analyzing SNAP25 migration in purified cytosol and membrane fractions, we can be confident that the number of SNAP25 molecules that are palmitoylated is increased by DHHC coexpression.

Cysteine Residues and Flanking Hydrophobic Amino Acids Are Important for SNAP25B Membrane Interactions

The previous section clearly demonstrated that the expression level of specific DHHC proteins regulates stable membrane binding of SNAP25B in HEK293 cells. However, as DHHC proteins are membrane associated, these results do not offer any insight into the mechanism of initial membrane interaction of SNAP25B. Syntaxin 1 has been proposed by several groups to regulate membrane binding of SNAP25, and indeed the two proteins were reported to interact in the cytosol of PC12 cells after synthesis (Vogel *et al.,* 2000). However, other groups have suggested that SNAP25 membrane trafficking occurs independently of syntaxin (see e.g., Gonzalo *et al.,* 1999; Loranger and Linder, 2002; Medine *et al.,* 2007), and we have shown that DHHC proteins are sufficient to drive stable membrane attachment of SNAP25 in HEK293 cells in the absence of syntaxin 1 expression. The major isoform of syntaxin 1 in PC12 cells is syntaxin 1A, and to directly examine the role of this protein in SNAP25 membrane binding in PC12 cells, we used siRNA specific for syntaxin 1A to deplete cellular expression levels. Reduction of syntaxin 1A by ~70% (Figure 2A) was found to have no effect on SNAP25 membrane association (Figure 2B), despite reports that endogenous SNAP25 is expressed at several-fold excess above syntaxin 1 in this cell type (Xiao *et al.,* 2004).

The lack of a detectable effect of syntaxin 1A knockdown on SNAP25 membrane association in PC12 cells is consistent

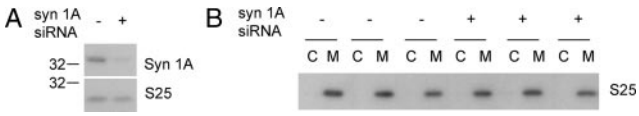

Figure 2. Knockdown of syntaxin1A expression in PC12 cells does not affect membrane binding of endogenous SNAP25. PC12 cells were incubated in the presence of 100 nM random siRNA (−) or syntaxin 1A siRNA (+) for ~ 70 h. Cells were then either lysed directly in SDS-dissociation buffer (A) of fractionated into cytosol (C) and membrane (M) fractions (B). Recovered fractions were analyzed by immunoblotting using antibodies against syntaxin 1A or SNAP25 as indicated. Position of molecular-weight standards are indicated on the left.

with the finding that DHHC expression in HEK293 cells (which do not express endogenous syntaxin 1) is sufficient to drive stable membrane binding of SNAP25 (Figure 1). On the basis of our recent results examining membrane binding and palmitoylation of CSP (Greaves and Chamberlain, 2006; Greaves *et al.,* 2008), we reasoned that perhaps the hydrophobicity of cysteine residues is important for initial membrane contact of SNAP25B. A number of studies have examined the effects on SNAP25 membrane interactions of mutating individual cysteine residues to alanine, serine, or glycine. The general consensus from these studies is that mutation of an individual cysteine reduces membrane binding/palmitoylation by ~50%, whereas mutation of any two cysteines results in an ~90% reduction (Lane and Liu, 1997; Gonelle-Gispert *et al.,* 2000; Washbourne *et al.,* 2001). However, no comprehensive analysis of the individual cysteine residues has been performed in a cell type that endogenously expresses SNAP25. Given that SNAP25 has four potential palmitoylation sites and that (in simplistic terms, at least) stable membrane binding should be bestowed by two palmitates (Shahinian and Silvius, 1995), it is not clear why individual cysteine mutations have such a large effect on membrane binding. A possibility could be that the presence of multiple cysteines increases the likelihood that one cysteine will be palmitoylated, which would enhance membrane affinity and subsequently increase the likelihood that other cysteines will be modified. Notwithstanding this, one possible problem with previous analyses is that alanine, serine, and glycine are all less hydrophobic than cysteine, and hence defects in membrane binding caused by such mutations could reflect an inhibition of initial hydrophobic membrane interactions rather than a loss of cysteine palmitoylation.

To test this idea, we individually mutated each cysteine residue in SNAP25B (C85, C88, C90, and C92) to either alanine or leucine. Alanine is less hydrophobic than cysteine, whereas leucine has a similar (or greater) hydrophobicity. These experiments were performed in PC12 cells, in which EGFP-SNAP25B is efficiently palmitoylated and trafficked (see e.g., Salaun *et al.,* 2005). Interestingly, for the alanine substitutions, we observed a marked difference in the effect on membrane binding dependent on which cysteine was mutated. C85A and C88A mutations decreased membrane binding by >50%, whereas C92A had a lesser effect and C90A was almost without effect on membrane interaction (Figure 3B). Thus, the individual cysteines each contribute to stable SNAP25 membrane interaction to different extents. Intriguingly though, the effect of every alanine substitution was almost completely reversed by replacement with leucine (Figure 3B). These results are consistent with an important role for cysteine hydrophobicity in SNAP25B membrane interactions; we propose that this role is to facil-

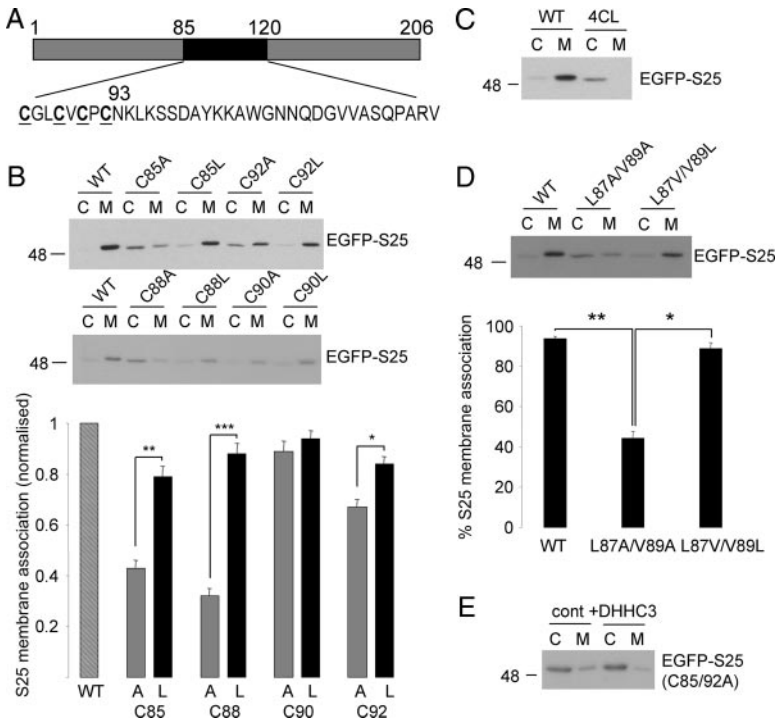

Figure 3. Membrane binding of SNAP25B proteins with mutations in the cysteine-rich domain. (A) Schematic diagram of SNAP25B with the minimal membrane-targeting sequence (residues 85-120) shown in black. The sequence of amino acids 85-120 is given, with cysteine residues highlighted in bold and underlined. (B) C85, C88, C90, and C92 in full-length SNAP25B fused to the C-terminus of EGFP were mutated individually to alanine or leucine residues. The mutant constructs were transfected into PC12 cells, and ~40 h later the cells were fractionated into cytosol (C) and membrane (M) fractions. Distribution of the cysteine mutants in the recovered fractions was analyzed by immunoblotting with anti-GFP. Top panel, representative immunoblots; bottom panel, averaged data for membrane binding; error bars, SE (n = 5 for C85A, C85L, C88A, C88L, C90A, and C90L; n = 6 for C92A and C92L). The level of membrane binding of the C85L, C88L,and C92L mutants was statistically different from the C85A, C88A, and C92A mutants, respectively: * p of < 0.005 (Student's *t* test), ** $p < 0.0001$, and *** $p < 0.00001$. (C) Distribution of SNAP25B wild-type and 4CL mutant in cytosol (C) and membrane (M) fractions from PC12 cells. (D) Distribution of SNAP25B wild-type, L87A/V89A, and L87V/V89L mutants in cytosol (C) and membrane (M) fractions purified from transfected PC12 cells. Top panel, representative immunoblot; bottom panel, averaged data for the % membrane binding (n = 3). The level of membrane binding of the L87A/V89A mutant was significantly reduced compared with wild-type SNAP25 (** $p < 0.00007$) and compared with L87V/V89L (* $p < 0.002$), and there was no significant difference between wild-type SNAP25B and the L87V/V89L mutant. (E) HEK293 cells were transfected with EGFP-SNAP25B(C85/C92A) mutant with or without DHHC3 cotransfection, and ~20 h later the cells were fractionated into cytosol (C) and membrane (M) fractions, which were analyzed by immunoblotting using anti-GFP. The position of molecular-weight standards are shown on the left side of all figure parts.

itate initial membrane interaction and thus ensure spatial proximity of the cysteines to specific membrane-localized DHHC palmitoyl transferases. As a control to ensure that the introduction of leucine residues to the cysteine-rich domain was not creating an artificial membrane-binding domain, all four cysteine were replaced with leucines (4CL). Figure 3C shows that the SNAP25B(4CL) mutant did not associate appreciably with cell membranes. Thus, although leucines can substitute for individual cysteines, introduction of leucines is not sufficient to promote membrane association in the absence of palmitoylation. The proposal that cysteine hydrophobicity is important for initial membrane association of SNAP25 is also strengthened by the observation that amino acids surrounding the palmitoylated cysteines are predominantly hydrophobic (see Figure 3A). Indeed, the introduction of a double L87A/V89A mutation also significantly inhibited SNAP25 membrane binding (Figure 3D), an effect that was reversed when the hydrophobic character of these amino acids was maintained (L87V/V89L). Thus, the hydrophobicity of the cysteine-rich domain as a whole plays an important role in SNAP25 membrane binding.

As previously shown, we also found that a double cysteine mutant (C85A/C92A) reduced membrane binding of SNAP25B by ~90% in PC12 cells (data not shown). To test the proposal that this dramatic loss of membrane binding of C85A/C92A reflects a loss of initial membrane targeting, we tested whether coexpression of DHHC3 could rescue membrane binding of this mutant in HEK293 cells. The C85A/C92A mutant was present almost entirely in the cytosolic fraction of HEK293 cells and this distribution did not change upon DHHC3 coexpression (Figure 3E). As the C85A/C92A mutant still retains two palmitoylation sites, this result is consistent with a loss of initial membrane binding of C85A/C92A and hence inability to localize in proximity to the DHHC3 protein.

Mutational Analysis of the Role of Amino Acids 93-120 in SNAP25B Membrane Binding

The data presented in the previous section support the notion that cysteine residues in SNAP25B play an important role in initial membrane interaction of SNAP25B. However, previous work, analyzing SNAP25 truncation mutants, mapped the minimal membrane-targeting sequence of SNAP25B to residues 85-120 (Gonzalo *et al.*, 1999), which includes the palmitoylated cysteine residues at positions 85, 88, 90, and 92 (Figure 3A) and the downstream 28 amino acids. It is not clear whether residues 93-120 play a direct role in membrane binding of *full-length* SNAP25B (e.g., by forming an essential part of the DHHC-binding site). As a first step to dissect the role of residues 93-120 in membrane binding of SNAP25B, we examined the possibility that residues downstream of the palmitoylated cysteines mediate initial membrane interactions of SNAP25B. Thus, PC12 cells were transfected with S25(85-120)-EGFP or EGFP-S25(93-120) constructs, and the protein distribution was analyzed by confocal imaging. Figure 4A shows that, in contrast to the S25(85-120) construct that was efficiently targeted to the plasma membrane (Gonzalo *et al.*, 1999), the S25(93-120) construct showed a dispersed localization that was distributed throughout the cytoplasm and the nucleus of PC12 cells. These data demonstrate that region 93-120 of SNAP25B does not contain strong autonomous membrane-targeting information.

As amino acids 93-120 of SNAP25B lack obvious membrane-targeting signals, we next examined the role of this domain in membrane binding of full-length SNAP25B using

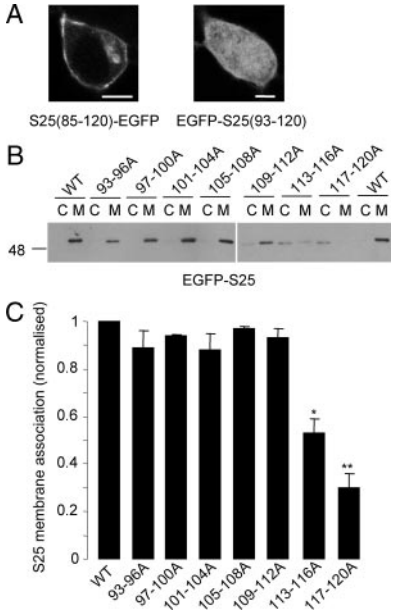

Figure 4. Mutational analysis of SNAP25B membrane targeting. (A) The distribution of S25(85-120)-EGFP and EGFP-S25(93-120) constructs in PC12 cells was examined by confocal imaging. Scale bars, 5 μm. (B) Residues 93-120 present within full-length SNAP25B fused to the C-terminus of EGFP were mutated in blocks of four amino acids to alanine (any alanines already present in this region were mutated to leucine). The constructs were transfected into PC12 cells, and ~40 h later the cells were fractionated into cytosol (C) and membrane (M) fractions. The distribution of the EGFP-tagged proteins in isolated fractions was examined by immunoblotting with anti-GFP. The position of molecular-weight standards are shown on the left side. (C) The relative levels of the mutant proteins in cytosol and membrane fractions were quantified by densitometry and expressed as % membrane association. The graph shows averaged data (n = 3); error bars, SE. The level of membrane association of 113-116A and 117-120A were statistically different from wild-type SNAP25B: * $p < 0.005$ (Student's *t* test), ** $p < 0.002$.

an alanine-scanning mutagenesis approach. This represents the first analysis of the role of these amino acids in the trafficking of full-length SNAP25B and is important because it has been questioned whether trafficking of the isolated 85-120 domain faithfully represents trafficking of wild-type SNAP25B (Vogel *et al.*, 2000). Residues 93-120 were mutated in blocks of four amino acids to alanine (any alanines were mutated to leucine). The constructs were transfected into PC12 cells, and cytosol and membrane fractions prepared. As shown in Figure 4, B and C, the majority of amino acids between residues 93-120 were dispensable for membrane binding, with the exception of the C-terminal eight amino acids (residues 113-120). We also confirmed these results using a cell homogenization–based fractionation approach (Supplementary Figure S1). These results agree well with the work of Linder's group studying SNAP25B fragments, which showed a decrease in membrane binding when amino acids Q116, P117, and R119 were mutated together within the context of the SNAP25B(85-120) construct (Gonzalo *et al.*, 1999). Note that we consistently observed a lower expression level of the 113-116A and 117-120A mutants compared with wild-type EGFP-SNAP25B in PC12 cells, suggesting that turnover of these mutants may be increased, perhaps because of a loss of membrane binding. As an independent measure of membrane targeting and to detect any changes in intracellular localization of the various SNAP25B mutants, we also examined transfected cells by confocal imaging. All membrane-bound mutants (93-96A, 97-100A, 101-104A, 105-108A, and 109-112A) were strongly enriched at the plasma membrane (Figure 5), demonstrating that these amino acids are not required for either membrane binding or intracellular sorting of SNAP25B. Note that SNAP25B localizes to the plasma membrane and also to an intracellular endosome compartment, as described by Martin's group (Aikawa *et al.*, 2006). The relative level of SNAP25B in this intracellular compartment varied between individual cells, but we did not detect any consistent differences in the intracellular localizations of any of the membrane-bound mutants. In contrast, the 113-116A and 117-120A mutants both displayed a more dispersed cytosolic localization (Figure 5), although some association with the plasma membrane and intracellular membranes was still apparent. Thus, these results are in agreement with the subcellular fractionation data (Figure 4, B and C). These results do not support the proposal that the trafficking of the isolated 85-120 fragment might use a distinct mechanism from full-length SNAP25B (Vogel *et al.*, 2000).

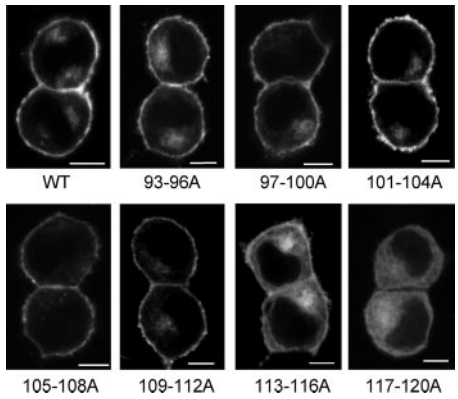

Figure 5. Intracellular localization of EGFP-SNAP25B proteins mutated within the minimal membrane-targeting sequence. The indicated plasmids were transfected into PC12 cells, and ~40 h later the distribution of expressed proteins was examined by confocal imaging. Note that all constructs with the exception of 113-116A and 117-120A were targeted efficiently to the plasma membrane. Scale bars, 5 μm.

To identify the specific amino acids within residues 113-116 and 117-120 that perturb membrane interactions when mutated, we examined the extent of membrane binding of individual alanine mutants. Mutation of V113 or Q116 each had a small but significant effect on membrane binding of SNAP25B (Figure 6, A and B). We presume that the combined effects of these mutations produced the greater defect in membrane binding observed with the 113-116A block mutant. In contrast to the relatively small effects of mutating V113 and Q116, the membrane-binding defect observed for the 117-120A block mutant was largely attributable to mutation of P117 (Figure 7A), suggesting that this residue plays a key role in membrane interactions of SNAP25B. These results clearly emphasize the importance of P117, and to a lesser extent V113 and Q116, in membrane interactions of full-length SNAP25B. Finally, we tested whether P117 was essential for membrane binding or if more conservative substitutions of this amino acid were tolerated. Thus, P117 was also mutated to valine, which is more representative of proline in terms of bulkiness and hydrophobicity (Wimley *et al.*, 1996; Wimley and White, 1996). Interestingly, we found that this P117V mutation had no major effect on SNAP25B

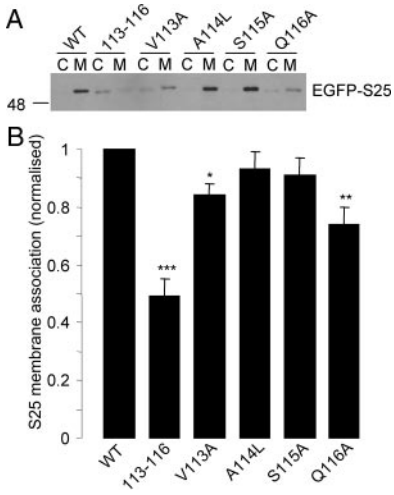

Figure 6. Analysis of membrane binding of EGFP-SNAP25B proteins containing point mutations within the amino acid region 113-116. The indicated constructs were transfected into PC12 cells, and ~40 h later the cells were fractionated into cytosol (C) and membrane (M) fractions. Distribution of the mutant proteins within the fractions was determined by immunoblotting with anti-GFP. (A) Representative immunoblot with position of molecular-weight standard indicated; (B) averaged data for % membrane association; error bars, SE (n = 3). The level of membrane binding of 113-116A, V113A, and Q116A were statistically different from wild-type SNAP25B: * $p < 0.05$ (Student's *t* test), ** $p < 0.02$, and *** $p < 0.002$.

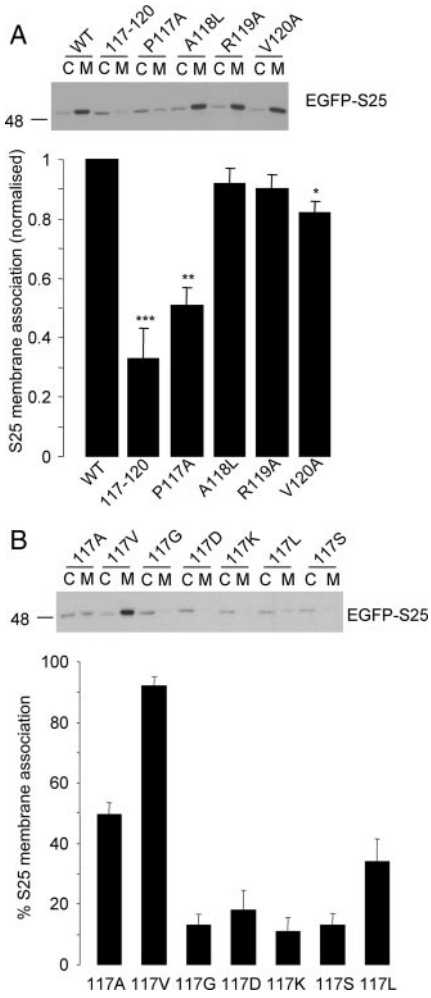

Figure 7. Analysis of membrane binding of EGFP-SNAP25B proteins containing point mutations within the amino acid region 117-120. (A) The indicated constructs were transfected into PC12 cells, and ~40 h later the cells were fractionated into cytosol (C) and membrane (M) fractions. Distribution of the mutant proteins within the fractions was determined by immunoblotting with anti-GFP. Top panel, representative immunoblot; bottom panel, averaged data for % membrane binding; error bars, SE (n = 3). The level of membrane binding of 117-120A, P117A, and V120A were statistically different from wild-type SNAP25B: * $p < 0.03$ (Student's *t* test), ** $p < 0.01$, and *** $p < 0.002$. (B) Distribution in PC12 cell cytosol (C) and membrane (M) fractions of SNAP25 proteins with different amino acid substitutions introduced at proline-117. Top panel, representative immunoblot with position of molecular-weight marker indicated; bottom panel, averaged data for % membrane binding (n = 5).

membrane binding (Figure 7B). In contrast, replacing P117 with glycine, aspartic acid, lysine, leucine or serine all significantly inhibited membrane binding (Figure 7B).

Importance of the Spacing Between Cysteine Residues and Downstream Elements in the Membrane Targeting Domain for Efficient Membrane Binding of SNAP25B

Mutational analyses presented thus far have highlighted the importance of cysteine residues as well as downstream amino acids (in particular P117) for membrane binding of SNAP25B. We next investigated whether these two regions of the SNAP25 membrane-targeting domain are coupled. For this, a series of deletion mutants were constructed, in which a number of amino acids were removed between the cysteine-rich domain and P117. The specific amino acids that were removed were chosen because they were found to be nonessential for SNAP25B membrane binding (Figure 4) and because they were roughly equidistant from the two domains of interest. Removal of amino acids 101–104 or 105–108 had no detectable effect on membrane binding of EGFP-SNAP25B (Figure 8A). When longer deletions were constructed, we found that the removal of seven amino acids (Δ101–107) caused a marked loss of SNAP25B membrane binding (Figure 8B). These results show that the spacing between the cysteine-rich domain and residues downstream (P117) is important, suggesting that these two regions of the membrane-targeting domain are functionally coupled.

Differential Effects of Specific DHHC Proteins on Membrane Binding of SNAP25B Mutants

We used the ability of DHHC enzymes to rescue SNAP25B membrane association in HEK293 cells to probe the role of residues 117-120 of SNAP25B in stable membrane binding; for example, one possibility would be that these residues are important for DHHC recognition. For this, SNAP25B(117-120A) and SNAP25B(Δ101–107) were transfected into HEK293 cells either with DHHC3, DHHC7, DHHC17, or empty vector. Recovered cytosol and membrane fractions were resolved on 10% SDS-PAGE gels to allow visualization of the palmitoylation-dependent band-shift. Results presented (Figure 9) clearly show that both DHHC3 and DHHC7 enhanced membrane binding of the 117-120A and Δ101–107 mutants and also that a band-shift occurs for membrane-bound protein, consistent with palmitoylation. In contrast, cotransfection of DHHC17 only very weakly stimulated membrane binding of the SNAP25 mutants (Figure 9), suggesting that residues 117-120 and the spacing of these residues from the cysteine-rich domain are particu-

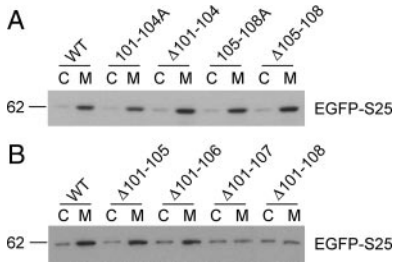

Figure 8. Effects of amino acid deletions in the membrane-targeting domain of SNAP25B on membrane binding. (A and B) PC12 cells were transfected with EGFP-SNAP25B constructs containing the indicated mutations within the membrane-targeting domain. After ~40 h of transfection, the cells were fractionated into cytosol (C) and membrane (M) fractions, which were analyzed by immunoblotting with anti-GFP. Position of molecular-weight standards are shown on the left side of A and B.

larly important for recognition of SNAP25B by DHHC17. Note that experiments with wild-type SNAP25 were performed in parallel, confirming that DHHC17 is active against wild-type SNAP25B under identical conditions (data not shown).

Finally, we used [^{3}H]palmitate-labeling experiments to directly examine palmitoylation of wild-type SNAP25 and the 117-120A mutant by coexpressed DHHC3 and DHHC17. Figure 9C shows that wild-type SNAP25 was robustly palmitoylated when coexpressed with DHHC3 and DHHC17. In contrast, the 117-120A mutant was efficiently palmitoylated by DHHC3 but not detectably by DHHC17.

DISCUSSION

On the basis of the results of this study, we propose that specific DHHC proteins mediate stable membrane binding of SNAP25B. The pathway involved in SNAP25 membrane binding has been a controversial area of investigation, and in particular it has been debated over a number of years whether syntaxin 1 regulates initial membrane interactions of SNAP25 (Gonzalo *et al.*, 1999; Vogel *et al.*, 2000; Washbourne *et al.*, 2001; Loranger and Linder, 2002). Our results clearly show that membrane interaction of SNAP25B in HEK293 cells is not inefficient because of low expression of syntaxin isoforms, but instead most likely results from a paucity of specific SNAP25B-palmitoylating enzymes. The finding that DHHC proteins mediate membrane binding of SNAP25B is particularly relevant as, unlike tail-anchored proteins such as syntaxin 1, DHHC proteins are polytopic membrane proteins that are membrane-inserted cotranslationally. This essentially rules out a coordinated interaction of newly synthesized DHHC and SNAP25B in the cytosol that would facilitate initial membrane targeting of SNAP25B.

Mutational analyses of SNAP25B membrane binding are consistent with the notion that membrane association of unpalmitoylated SNAP25B is mediated by hydrophobic interactions of the cysteine-rich domain with the membrane. Replacement of single cysteines with alanine residues had a marked effect on membrane binding, particularly when C85 or C88 were substituted. However, membrane binding was restored when these cysteines were replaced by more hydrophobic leucine residues, demonstrating that cysteines are not just sites for palmitate attachment but that other features of the cysteine residues (presumably hydrophobicity) also contribute to membrane binding. The finding that C85A and

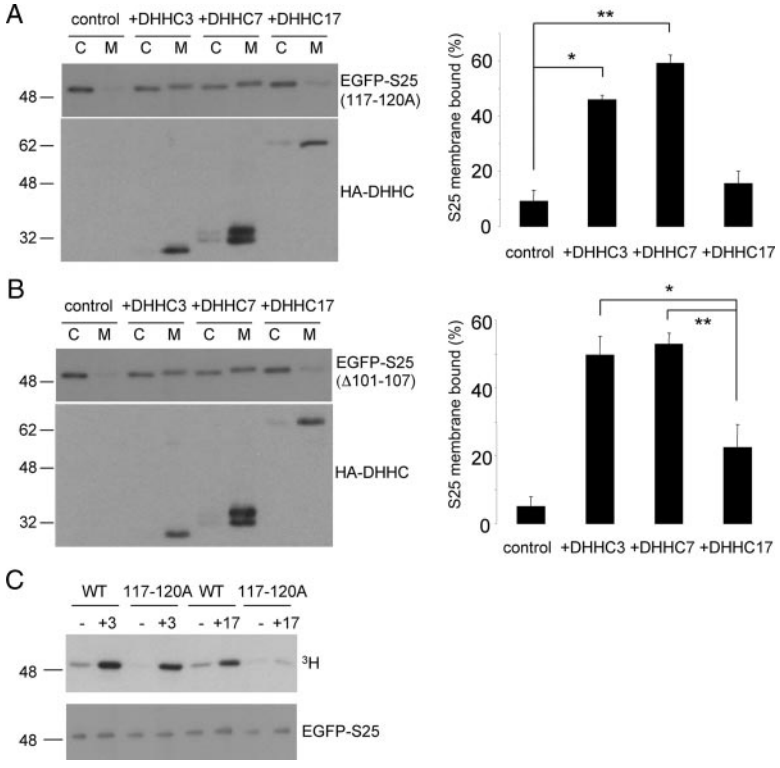

Figure 9. Distinct effects of coexpression of different DHHC proteins on membrane association and palmitoylation of SNAP25B mutants. HEK293 cells were transfected with SNAP25B(117-120A; A) or SNAP25B(Δ101–107; B) plasmids in the presence of DHHC3, DHHC7, DHHC17, or empty vector (control). After ~20 h of transfection, the cells were fractionated into cytosol (C) and membrane (M) fractions, which were probed by immunoblotting with anti-GFP and anti-HA antibodies. Left panels, representative immunoblots with the position of molecular-weight standards highlighted on the left side; right panels, averaged data (n = 4) for % membrane binding of the SNAP25B mutants when transfected in the presence of the indicated DHHC plasmids compared with SNAP25B transfected with empty vector. (A) * $p < 0.00006$ and ** $p < 0.00003$ compared with membrane binding of SNAP25B(117-120A) in the absence of DHHC expression. There was no significant difference in the level of membrane binding of SNAP25B(117-120A) in the absence of DHHC expression or after coexpression with DHHC17 ($p = 0.26$). (B) Although DHHC17 significantly increased membrane binding of SNAP25B(Δ101–107), the effect of DHHC17 coexpression on membrane association of SNAP25B(Δ101–107) was significantly reduced compared with DHHC3 (* $p < 0.01$) and DHHC7 (** $p < 0.003$) coexpression. (C) HEK293 cells were transfected with wild-type or 117-120A mutant SNAP25B in the absence (−) or presence of DHHC3/DHHC17 coexpression. Approximately 20 h after transfection, the cells were labeled with [^{3}H]palmitic acid for 4 h, and the EGFP-tagged SNAP25 proteins then were immunoprecipitated. [^{3}H]palmitate incorporation was assessed using an enhancer screen, and levels of immunoprecipitated proteins were determined by immunoblotting with anti-GFP.

C88A mutations had a larger effect on membrane binding than C90A and C92A mutations is consistent with previous work studying palmitoylation and membrane binding in Cos-7 cells, which showed that C85S and C88S mutations resulted in a greater loss in SNAP25B palmitoylation and membrane binding than mutation of C90S or C92S (Lane and Liu, 1997). The cysteine residues in the cysteine-rich domain of SNAP25 are part of an overall hydrophobic domain, and we also found that L87 and V89, which sit adjacent to C88, are important for SNAP25 membrane interaction. In a similar manner to the cysteine residues, the hydrophobicity of L87 and V89 appears to be important for membrane binding, as alanine mutation of these residues inhibits membrane binding, whereas switching the residues (and hence maintaining hydrophobicity) does not have a deleterious effect on membrane interaction. It was interesting that individual cysteine-to-leucine mutants never restored SNAP25 membrane binding exactly to wild-type levels. This might suggest that the presence of multiple closely spaced cysteines plays an additional role in stable membrane binding by increasing the likelihood that palmitoylation of a cysteine residue will occur, and this is likely to be important for proteins with a weak membrane affinity such as SNAP25.

A role for cysteine residues and flanking hydrophobic amino acids in regulating initial membrane interaction of SNAP25 agrees with our previous work implicating cysteine hydrophobicity in initial membrane attachment of CSP (Greaves and Chamberlain, 2006). This close association of cysteine-rich domains with the membrane is an attractive idea as it would ensure that cysteines are in intimate membrane contact, which appears to be an important factor in determining palmitoylation sites. It is not clear whether SNAP25B is self-sufficient for initial membrane interaction or if additional "chaperones" regulate movement of the newly synthesized protein to membranes. If additional molecules are required, then these are likely to be widely expressed (as they are not limiting in HEK293 cells). Thus, we propose that SNAP25B utilizes a similar mechanism of membrane binding/palmitoylation as previously proposed by us for CSP (Greaves and Chamberlain, 2006; Greaves *et al.*, 2008). One interesting difference, however, is that CSP and SNAP25B membrane binding differ in their sensitivity to brefeldin A (BFA; Gonzalo and Linder, 1998; Greaves *et al.*, 2008); membrane binding of SNAP25B is sensitive to BFA, whereas CSP is resistant. As DHHC proteins retain their activity after BFA treatment of both PC12 and HEK293 cells (Greaves *et al.*, 2008), we propose that this difference in sensitivity might reflect a difference in initial membrane interactions; for example, SNAP25B might have a high affinity for intact Golgi membranes, whereas CSP has a more general membrane affinity.

Ras proteins have been suggested to undergo a dynamic cycle of palmitoylation and depalmitoylation that regulates localization between the plasma membrane and intracellular membranes (Goodwin *et al.*, 2005; Rocks *et al.*, 2005), and a similar cycle was proposed for other palmitoylated peptides (Rocks *et al.*, 2005). In this cycle, depalmitoylation of plasma membrane–localized protein results in release into the cytosol, where the protein can bind to intracellular membranes and be repalmitoylated and transported again to the plasma membrane. Such a palmitoylation cycle could be an intriguing pathway to regulate SNAP25 localization and hence exocytosis efficiency. However, although the half-life of palmitate attachment to SNAP25 was suggested to be shorter than the half-life of the protein in PC12 cells (Lane and Liu, 1997), no turnover of palmitate on SNAP25 was detected in cortical neurons (Kang *et al.*, 2004). Thus, it is unclear at this stage whether a similar palmitoylation cycle might operate for SNAP25, but the membrane-binding properties of the hydrophobic cysteine-rich domain of SNAP25 could be well suited to coordinate such a cycle.

In addition to the cysteine-rich domain, stable membrane binding of SNAP25B in PC12 cells is also dependent on downstream residues (P117) and their spacing from the palmitoylated cysteines. P117 was also identified by Linder and coworkers as part of a group of amino acids important for trafficking of the isolated 85-120 membrane-targeting domain, and it was suggested that this region of SNAP25 might be important to allow binding to a palmitoyl transferase (Gonzalo *et al.*, 1999). The results presented here support these suggestions and, intriguingly, suggest that the region of SNAP25B containing P117 may also play an important role in determining DHHC specificity: at similar DHHC expression levels, this region of SNAP25B was important for palmitoylation and stable membrane binding induced by DHHC17 but not DHHC3 or DHHC7. This observation to our knowledge represents the first description of intrinsic substrate elements that might modulate DHHC specificity. At this stage we do not know why P117 is particularly important for efficient palmitoylation by DHHC17. The most obvious possibility is that this region of SNAP25 supports the direct interaction of SNAP25 and DHHC17. Alternatively, P117 may be important in promoting the association of SNAP25 with specific membranes or membrane domains that sequester DHHC17. Although DHHC3, DHHC7, and DHHC17 are all localized to the Golgi in HEK293 cells (Greaves *et al.*, 2008), it is not known whether the proteins are associated with the same or different Golgi cisternae. Similarly, it is not known whether these DHHC proteins are present in the same Golgi subdomains; for example, the ankyrin repeat region of DHHC17 might target the protein to actin-rich regions of the Golgi. Thus, P117 may also be important in ensuring efficient targeting of SNAP25B to the same membrane compartment or subcompartment that DHHC17 resides in.

The reduced membrane binding of the 117-120A and Δ101–107 mutants in PC12 cells is consistent with an important role for DHHC17 in regulating SNAP25 palmitoylation in this cell type. Attempts to deplete DHHC proteins by siRNA in PC12 cells have thus far proved unsuccessful. However, it is worth noting that depletion of DHHC17 in *Drosophila* was recently reported to cause mislocalization of SNAP25 (Ohyama *et al.*, 2007; Stowers and Isacoff, 2007), supporting the notion that DHHC17 regulates SNAP25 palmitoylation in vivo.

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